

Human P[6] Rotaviruses From Sub-Saharan Africa and Southeast Asia Are Closely Related to Those of Human P[4] and P[8] Rotaviruses Circulating Worldwide

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Background. P[6] rotaviruses have been circulating with a high prevalence in African and, to a more limited extent, Asian countries, but they have not been highly prevalent in other parts of the world.

Methods. To investigate the genomic relationship between African and Asian human P[6] rotaviruses and P[4] and P[8] rotaviruses circulating worldwide, we sequenced 39 P[6] strains, collected in Ghana, Mali, Kenya and Bangladesh, providing the largest data set of P[6] rotavirus genomes isolated in low-income countries or anywhere else in the world that has been published thus far.

Results. Overall, the data indicate that the genetic backbone of human P[6] strains from the low-income countries are similar to those of P[4] or P[8] strains circulating worldwide.

Conclusions. The observation that gene segment 4 is the main differentiator between human P[6] and non-P[6] strains suggests that the VP4 spike protein is most likely one of the main reasons preventing the rapid spread of P[6] strains to the rest of the world despite multiple introductions. These observations reinforce previous findings about the receptor specificity of P[6] rotavirus strains.

Keywords. group A rotavirus; vaccine-preventable diseases; epidemiology; gastrointestinal disease; zoonosis; P[6] genotype; complete genome analyses; clinical trial samples.

Group A rotavirus is the most important causative agent of gastroenteritis in both infants and young animals worldwide, resulting in a high mortality and burden of disease, mainly in low-income countries [1]. However, because the incidence of rotavirus gastroenteritis is similar in both high- and low-income countries, it is unlikely that improvements in hygiene alone will reduce the burden of disease. Fortunately, the global burden of disease in humans has dropped dramatically since the introduction of rotavirus vaccines in the infant immunization schedules of most countries [2]. There are currently 2 human oral rotavirus vaccines licensed worldwide: the human attenuated rotavirus vaccine (Rotarix) and the pentavalent rotavirus vaccine (PRV; RotaTeq). The latter contains human-bovine reassortant strains

and both vaccines have proven to be safe and efficacious in preventing severe gastroenteritis due to rotavirus in European and American regions [3, 4]. However, both vaccines are less efficacious in preventing severe gastroenteritis due to rotavirus in Africa and Asia [5]. For example, the vaccine efficacy in clinical trials conducted between March 2007 and March 2009 in Ghana, Kenya, Mali, Vietnam, and Bangladesh was only 39.3% (95% confidence interval [CI], 19.1%–54.7%) [6–9]. To explain this observation, multiple factors have been suggested, such as host population-associated factors related to overall health conditions (eg, malnutrition, level of maternal antibodies, or coinfecting pathogens) or the circulation of different genotypes in low-income countries as compared to high-income countries [10]. A full genome-based classification of rotavirus consists of 11 different genotypes, one for each of the 11 rotavirus gene segments [11]. Based on the sequences of the 2 outer capsid proteins, the glycoprotein VP7 and the spike-protein VP4, G and P genotypes can be determined. Especially the P[6] genotype seems to have a relative higher prevalence in low-income countries as compared to high-income countries, where P[6] strains are only sporadically detected [12–15]. The P[6] genotype is believed to be of porcine origin but is already circulating for decades in the human population [16]. The P[6] genotype is one of the 3 major P genotypes found in human rotaviruses, next to P[8] and P[4] [17]. However, the P[6] genotype is worldwide by far the least prevalent of the 3 VP4 genotypes and is genetically and antigenically more distantly related to P[8] and P[4] than P[8] and P[4] are

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related to each other [18, 19]. In general, P[8] and P[4] rotaviruses are found in combination with a Wa-like (genotype 1) and a DS-1-like (genotype 2) genotype constellation, respectively, while P[6] rotaviruses are found with both backgrounds [20].

Previous studies have suggested a link between susceptibility for rotavirus infections and the histo-blood group antigens phenotypes of the host [21, 22]. More specifically, the surface spike protein VP8* (one of the trypsin cleavage products of VP4) interacts with the secretor histo-blood group antigens that may function as ligands or receptors for rotavirus attachment to host cells. Depending on the P genotype, different histo-blood group antigen binding specificities were found, with P[4] and P[8] strains recognizing both the common Le^b as the H-type 1 antigens, while the P[6] rotaviruses exclusively recognize the H-type 1. Therefore, so-called nonsecretors—individuals who have an inactive α -1, 2 fucosyltransferase and therefore do not synthesize the H-type 1 antigen—are believed to be nonsusceptible to infection with P[6] rotaviruses. Complementary to this observation, Nordgren et al observed that P[6] rotaviruses preferentially infect Lewis-negative children [23]. The fact that the nonsecretor phenotype is found in about 20% of European and North American populations, while the number of nonsecretors is much lower in Africa, might explain why rotavirus strains with the P[6] genotype could have a selective evolutionary disadvantage outside Africa.

During phase 3 clinical trials of PRV in Ghana, Kenya, Mali, Vietnam, and Bangladesh, 24.3% (192) of 790 rotavirus-positive G- and P-typed fecal samples contained P[6] strains. Despite the epidemiological importance of these P[6] strains, previous epidemiological studies conducted in the clinical trial site countries (Mali, Ghana, Kenya, Bangladesh, and Vietnam) are scarce and are often based on multiplex instead of sequencing techniques for genotyping (Table 1). These studies have indicated a high prevalence of P[6] strains in all 5 countries, with a prevalence of up to 84.8% reported by Armah et al [48]. Little is known about the genetic composition of African or Asian P[6] rotavirus strains because only a small number of complete P[6] rotavirus genomes from these regions have been determined to date. However, in recent years more and more studies describing complete genomes of P[6] strains have been published. Some examples include studies conducted in Cameroon, Malawi, and Ghana, which reported 14, 11, and 2 complete P[6] rotavirus genomes, respectively [49–51].

In this study, we investigated the genomic relationship between human P[6] rotavirus strains and the more conventional non-P[6] rotaviruses (G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], and G12P[8]), to interpret the lower vaccine efficacy in low-income countries and to determine whether the decreased success of the P[6] strains to spread around the world can be explained by differences in their genetic background. Therefore, we determined the complete genome of 39 P[6] rotavirus strains isolated during the clinical trials of the PRV in Africa and Asia [8, 9], resulting in the largest data set known today of complete P[6] rotavirus genomes isolated in low-income countries.

METHODS

Study Design

The samples characterized in this study were collected during a randomized, placebo-controlled phase 3 trial (clinical trials registration NCT00362648) conducted between 28 April 2007 and 31 March 2009 in 5 different clinical trial sites. The 3 sites in sub-Saharan Africa were located in Kenya (Kenya Medical Research Institute, in collaboration with the Centers for Disease Control and Prevention), Ghana (Noguchi Memorial Institute for Medical Research, in collaboration with the Navrongo Health Research Centre), and Mali (Center for Vaccine Development, in collaboration with the University of Maryland). The 2 sites in Asia were located in Bangladesh (International Centre for Diarrhoeal Disease Research) and Vietnam (National Institute of Hygiene and Epidemiology; International Vaccine Institute). Detailed descriptions of the study design of the clinical trial have been reported elsewhere [6, 7]. Briefly, a subset of the rotavirus positive, G- and P-typed fecal specimens collected during the phase 3 clinical trial were selected by Merck and PATH for complete genome sequencing, based on the following criteria: (1) samples had to contribute to the per-protocol efficacy analysis (ie, cases had to occur >14 days after receipt of the third dose of PRV or placebo), (2) all observed G genotypes detected in combination with the P[6] genotype during the phase 3 clinical trial needed to be represented, (3) a sufficient stool sample with a positive enzyme-linked immunosorbent assay result had to be available, and (4) permission from central and local institutional review boards and local authorities to perform the analyses was granted. Unfortunately, rotavirus-positive samples from Vietnam could not be included in our study because of the lack of approval from the local medical ethical committees. Of the 192 P[6] rotavirus strains identified during the phase 3 clinical trials of PRV in sub-Saharan Africa and Southeast Asia, 39 P[6] rotavirus strains collected in Ghana ($n = 14$), Mali ($n = 18$), Kenya ($n = 2$), and Bangladesh ($n = 5$) were selected on the basis of the criteria mentioned above for full-genome sequencing. Of these 39 strains, 19 and 20 samples were isolated from study participants belonging to the placebo and vaccine groups, respectively (Table 1).

Sequencing and Data Analysis

Double-stranded RNA was extracted from 140 μ L of 20% raw stool suspensions, using the QIAamp Viral RNA mini-kit (Qiagen/Westburg, Leusden, the Netherlands) according to the manufacturer's instructions. Subsequently, RNA extracts were denatured at 95°C for 2 minutes (followed by cooling on ice), and reverse transcription–polymerase chain reaction (RT-PCR) analysis was performed using the Qiagen One Step RT-PCR kit (Qiagen/Westburg) on the Biometra T3000 thermocycler (Biometra, Westburg, the Netherlands). The initial RT step (50°C for 30 minutes) was followed by PCR activation (at 95°C for 15 minutes), 35 cycles of amplification (at 94°C for

Table 1. Overview of Epidemiological Studies Conducted in Humans in Ghana, Kenya, Mali, Vietnam, and Bangladesh in Which P[6] Rotavirus Strains Were Detected

Country, Period of Collection	Strains Recovered		G Genotypes Among P[6] Strain, No.												Gx	Mix	Reference(s)
	Overall, No.	P[6], No. (%)	1	2	3	4	5	8	9	10	11	12					
Bangladesh																	
Jan 2008–Sep 2009	50	11 (22)	0	1	0	0	0	0	1	0	0	9	0	0	[24]		
Apr 2007–Mar 2009	154	6 (3.9)	1	0	0	0	0	0	1	0	0	4	0	0	[8]		
Jun 2006–May 2012	1963	22 (1.1)	3	1	0	0	0	0	4	0	0	8	0	6	[25]		
2006–2009	166	20 (12)	3	1	0	0	0	0	9	0	0	7	0	0	[26]		
Aug 2005–Jul 2007	114	9 (7.9)	1	2	0	0	0	0	1	0	0	4	1	0	[27]		
Jul 2005–Jun 2006	145	24 (16.6)	2	1	0	0	0	0	0	0	0	12	4	5	[28]		
Jul 2004–Jun 2006	60	7 (11.7)	0	0	0	0	0	0	0	0	0	0	0	0	[29]		
2005–2006	30	3 (10)	0	0	0	0	0	0	0	0	0	0	0	0	[30]		
Jan 2005–Jun 2006	71	14 (19.7)	6	0	0	0	0	0	0	0	0	0	0	0	[31]		
2004–2005	284	1 (0.4)	0	0	0	0	0	0	1	0	0	0	0	0	[32]		
Jul 2004–Jun 2006	155	47 (30.3)	9	3	0	1	0	0	5	0	0	4	1	24	[33]		
Jan 2001–May 2006	456	35 (7.7)	3	1	0	0	0	0	9	0	1	21	0	0	[34]		
1992–1997	351	61 (17.4)	5	3	0	10	0	0	37	0	0	0	1	5	[35]		
Ghana																	
Jan 2004–Jan 2005	70	28 (40)	1	3	4	0	0	0	7	0	0	0	10	2	[36]		
Aug 2007–Feb 2011	624	218 (34.9)	23	45	56	5	0	0	14	23	0	1	35	16	[37, 38]		
Apr 2007–Mar 2009	138	39 (28.3)	0	16	15	0	0	8	0	0	0	0	0	0	[9]		
Nov 2005–Jan 2006	142	8 (5.6)	8	0	0	0	0	0	0	0	0	0	0	0	[39]		
Jul 1998–Mar 2000	238	70 (29.4)	7	43	6	0	0	6	8	0	0	0	0	0	[40]		
Jan 1999–Apr 1999	46	39 (84.8)	0	23	0	0	0	2	0	0	0	0	8	6	[41]		
Aug 1998–Dec 1998	50	12 (24)	1	5	3	0	0	0	0	0	0	0	0	3	[42]		
Kenya																	
Aug 2012–Jul 2013	36	7 (19.4)	0	0	0	0	0	0	5	0	0	0	0	2	[43]		
Sep 2009–Aug 2011	138	11 (22)	0	0	0	0	0	0	7	0	0	4	0	0	[44]		
Apr 2007–Mar 2009	89	25 (16.2)	2	0	0	0	0	21	2	0	0	0	0	0	[9]		
2002–2004	558	74 (13.3)	9	0	0	0	0	33	12	0	0	0	5	15	[45]		
2000–2002	108	17 (15.7)	3	1	0	0	0	5	2	0	0	0	1	5	[46]		
Jan 2000–Mar 2000	20	2 (10)	1	0	0	0	0	0	1	0	0	0	0	0	[47]		
Mali																	
Apr 2007–Mar 2009	366	120 (32.8)	22	81	0	0	0	17	0	0	0	0	0	0	[9]		

Abbreviation: Gx, undetermined G genotype.

30 seconds, at 50°C for 30 seconds, and at 72°C for 1.5 minutes [for VP7, VP6, NSP2, NSP3, NSP4, and NSP5] or 6 minutes [for VP1, VP2, VP3, VP4, and NSP1]), with a final extension at 72°C for 10 minutes. Primers used to amplify all gene segments were described elsewhere [52]. All 11 amplicons were pooled, taking into account the size of each gene segment and the yield obtained per RT-PCR analysis. Samples were sequenced on the 454 Roche GS-FLX sequencing platform. The sequence reads obtained were initially mapped against a VP7 and VP4 matching the already known G and P genotype of each sample completed with a Wa- or DS-1 background in Mira 3.4.0 or using the CLC Genomics Workbench 7.0. If necessary, a de novo assembly was performed. Sites with insufficient sequence read coverage after combining the reference mapping and the de novo assembly results were resequenced using traditional Sanger sequencing. The obtained consensus sequences were submitted to GenBank (accession numbers

are available in Figure 1) and aligned with a set of rotavirus genomes and manually edited for insertions and deletions in homopolymer regions. Nucleotide sequences for every gene segment were aligned together with those of strains from GenBank collected all over the world and were analyzed phylogenetically, using the maximum likelihood method with the best fit model in MEGA 6 [53]. Bootstrap resampling analysis (500 replicates) was performed to measure the reliability of the tree topologies.

RESULTS

Genetic analyses revealed that P[6] strains were associated with a wide range of G genotypes: G2 (n = 17), G3 (n = 7), G8 (n = 7), G12 (n = 5), G1 (n = 2), and G9 (n = 1). In Figure 1, the complete genotype constellation of all analyzed P[6] rotavirus strains collected during the phase 3 clinical trial in Africa and Asia is shown. The 7 P[6] strains associated with a G8 genotype

Strain Name	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5	GenBank Accession No.	Group
RVA/Human-wt/MLI/Mali-075/2008/G1P[6]	G1	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1	KP883110-KP883120	Placebo
RVA/Human-wt/MLI/Mali-120/2009/G1P[6]	G1	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP883143-KP883153	Vaccine
RVA/Human-wt/GHA/Ghan-053/2009/G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882428-KP882438	Placebo
RVA/Human-wt/GHA/Ghan-009/2009/G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882362-KP882372	Placebo
RVA/Human-wt/GHA/Ghan-060/2009/G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882483-KP882493	Vaccine
RVA/Human-wt/GHA/Ghan-108/2009/G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882604-KP882614	Placebo
RVA/Human-wt/GHA/Ghan-052/2008/G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882417-KP882427	Vaccine
RVA/Human-wt/MLI/Mali-028/2008/G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882824-KP882834	Vaccine
RVA/Human-wt/MLI/Mali-029/2008/G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882835-KP882845	Placebo
RVA/Human-wt/MLI/Mali-030/2008/G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882846-KP882856	Vaccine
RVA/Human-wt/MLI/Mali-035/2008/G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882890-KP882900	Vaccine
RVA/Human-wt/MLI/Mali-036/2008/G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882901-KP882911	Placebo
RVA/Human-wt/MLI/Mali-045/2009/G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882989-KP882999	Vaccine
RVA/Human-wt/MLI/Mali-069/2009/G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP883055-KP883065	Vaccine
RVA/Human-wt/MLI/Mali-070/2008/G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP883066-KP883076	Vaccine
RVA/Human-wt/MLI/Mali-071/2008/G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP883077-KP883087	Vaccine
RVA/Human-wt/MLI/Mali-072/2008/G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP883088-KP883098	Vaccine
RVA/Human-wt/MLI/Mali-074/2008/G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP883099-KP883109	Vaccine
RVA/Human-wt/MLI/Mali-104/2008/G2P[6]	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP883121-KP883131	Vaccine
RVA/Human-wt/GHA/Ghan-006/2009/G3P[6]	G3	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882329-KP882339	Placebo
RVA/Human-wt/GHA/Ghan-007/2009/G3P[6]	G3	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882340-KP882350	Placebo
RVA/Human-wt/GHA/Ghan-055/2009/G3P[6]	G3	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882450-KP882460	Vaccine
RVA/Human-wt/GHA/Ghan-056/2009/G3P[6]	G3	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882461-KP882471	Placebo
RVA/Human-wt/GHA/Ghan-107/2009/G3P[6]	G3	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882593-KP882603	Vaccine
RVA/Human-wt/GHA/Ghan-106/2009/G3P[6]	G3	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882582-KP882592	Vaccine
RVA/Human-wt/GHA/Ghan-105/2009/G3P[6]	G3	P[6]	I2	R2	C2	M2	A2	N1	T2	E2	H1	KP882571-KP882581	Placebo
RVA/Human-wt/KEN/Keny-061/2008/G9P[6]	G9	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H1	KP941126-KP941136	Vaccine
RVA/Human-wt/KEN/Keny-078/2008/G8P[6]	G8	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882714-KP882724	Placebo
RVA/Human-wt/MLI/Mali-039/2008/G8P[6]	G8	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882923-KP882933	Placebo
RVA/Human-wt/MLI/Mali-048/2008/G8P[6]	G8	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP883022-KP883032	Placebo
RVA/Human-wt/MLI/Mali-119/2008/G8P[6]	G8	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP883132-KP883142	Vaccine
RVA/Human-wt/MLI/Mali-135/2008/G8P[6]	G8	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP883176-KP883186	Placebo
RVA/Human-wt/GHA/Ghan-113/2008/G8P[6]	G8	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882626-KP882636	Placebo
RVA/Human-wt/GHA/Ghan-149/2008/G8P[6]	G8	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2	KP882681-KP882691	Placebo
RVA/Human-wt/BGD/Bang-014/2008/G12P[6]	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1	KP881900-KP881910	Placebo
RVA/Human-wt/BGD/Bang-015/2008/G12P[6]	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1	KP881911-KP881921	Vaccine
RVA/Human-wt/BGD/Bang-017/2008/G12P[6]	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1	KP881933-KP881943	Placebo
RVA/Human-wt/BGD/Bang-020/2008/G12P[6]	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1	KP881966-KP881976	Vaccine
RVA/Human-wt/BGD/Bang-129/2009/G12P[6]	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1	KP882230-KP882240	Placebo

Figure 1. Genotype constellation and GenBank accession numbers of selected P[6] rotavirus strains isolated in Ghana, Kenya, Mali, and Bangladesh. The red genotypes are of typical human DS-1-like rotavirus origin, the P[6] VP4 genotype is yellow, and blue is used to indicate gene segments of distinct animal (most likely bovine or similar) origin. In addition, green is used for Wa-like genotypes, and different shades of green are used for the VP7 G genotypes usually found with a Wa-like backbone. This figure is available in black and white in print and in color online.

have been analyzed in detail elsewhere, showing multiple gene segments of animal origin [52].

Analyses of the non-G8 P[6] rotaviruses showed that the large majority (30 of 32) possessed either a complete Wa-like

or DS-1-like backbone, with both exceptions containing Wa-like gene segments in a DS-1-like backbone. First, strain RVA/Human-wt/GHA/Ghan-105/2009/G3P[6] not only possessed the G3 genotype (usually associated with a Wa-like genotype constellation), but also contained both Wa-like NSP2 (N1) and NSP5 (H1) gene segments in a DS-1-like genetic background. Second, strain RVA/Human-wt/KEN/Keny-061/2008/G9P[6] possessed the G9 genotype (commonly found with a Wa-like genotype constellation), as well as Wa-like NSP5 (H1) gene segment, in a DS-1-like background.

The P[6] rotavirus strains isolated in Bangladesh (n = 5) had a complete Wa-like backbone (in combination with G12), while most rotavirus strains from sub-Saharan Africa were DS-1 like (in combination with G1, G2, G3, or G9). Only 1 African P[6] strain (RVA/Human-wt/MLI/Mali-075/2008/G1P[6]) was found to possess a complete Wa-like genotype constellation.

Seven entirely DS-1-like African rotavirus strains possessed a VP7 G genotype atypical for rotaviruses with a DS-1-like background, including 1 G1 and 6 G3 strains.

Figure 2A shows that the investigated African and Asian P[6] strains could be subdivided into 3 clusters based on geographical location of isolation: a West African cluster (containing rotaviruses from Ghana and Mali), a Kenyan cluster, and a Bangladeshi cluster. However, the genetic distance between each cluster was <5%, and all of them clustered closely to other human contemporary P[6] rotaviruses isolated all over the world. The P[6] strains analyzed in this study were found with a wide variety of different VP7 G genotypes, representing the most prevalent G genotypes in humans (Figure 2B). The only missing common human G genotype was G4, which was not detected during the clinical trial. The majority of P[6] strains analyzed in this study possessed the G2 genotype and

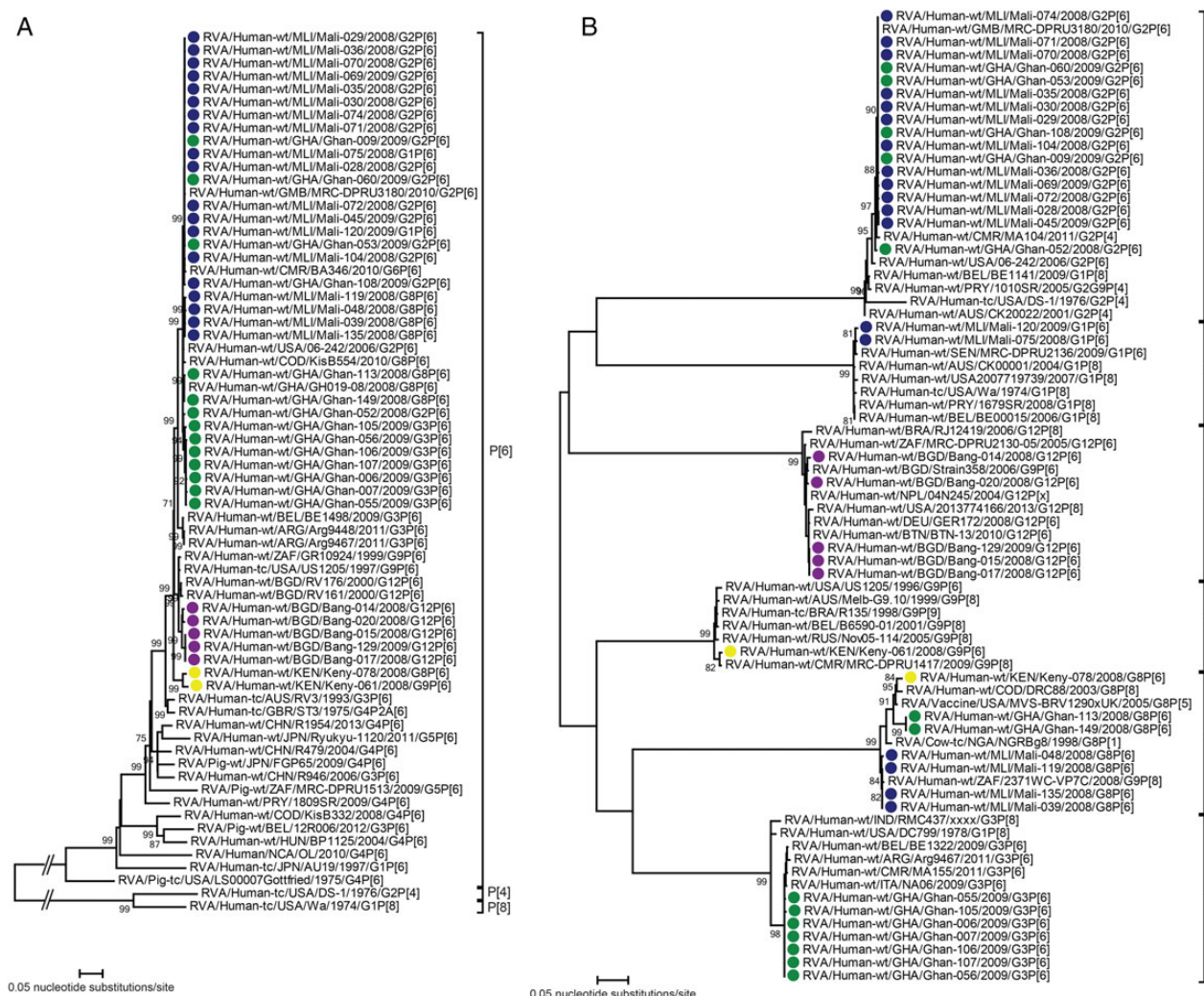
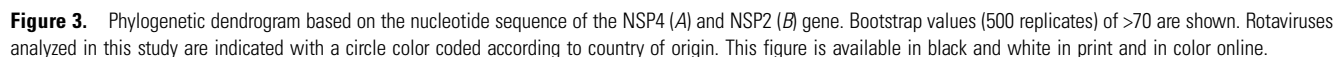


Figure 2. Phylogenetic dendrogram based on the nucleotide sequence of the VP4 (A) and VP7 (B) genes. Bootstrap values (500 replicates) of >70 are shown. Rotaviruses analyzed in this study are indicated with a circle color coded according to country of origin. This figure is available in black and white in print and in color online.

Figure 3A shows that the NSP4 gene segments of the 39 investigated P[6] strains clustered in NSP4 genotypes: E1 and E2.

The phylogenetic tree of NSP2 (Figure 3B) shows 7 strains (all 5 Bangladeshi strains, Mali-075, and Ghan-105) clustering

The phylogenetic tree of NSP2 (Figure 3B) shows 7 strains (all 5 Bangladeshi strains, Mali-075, and Ghan-105) clustering



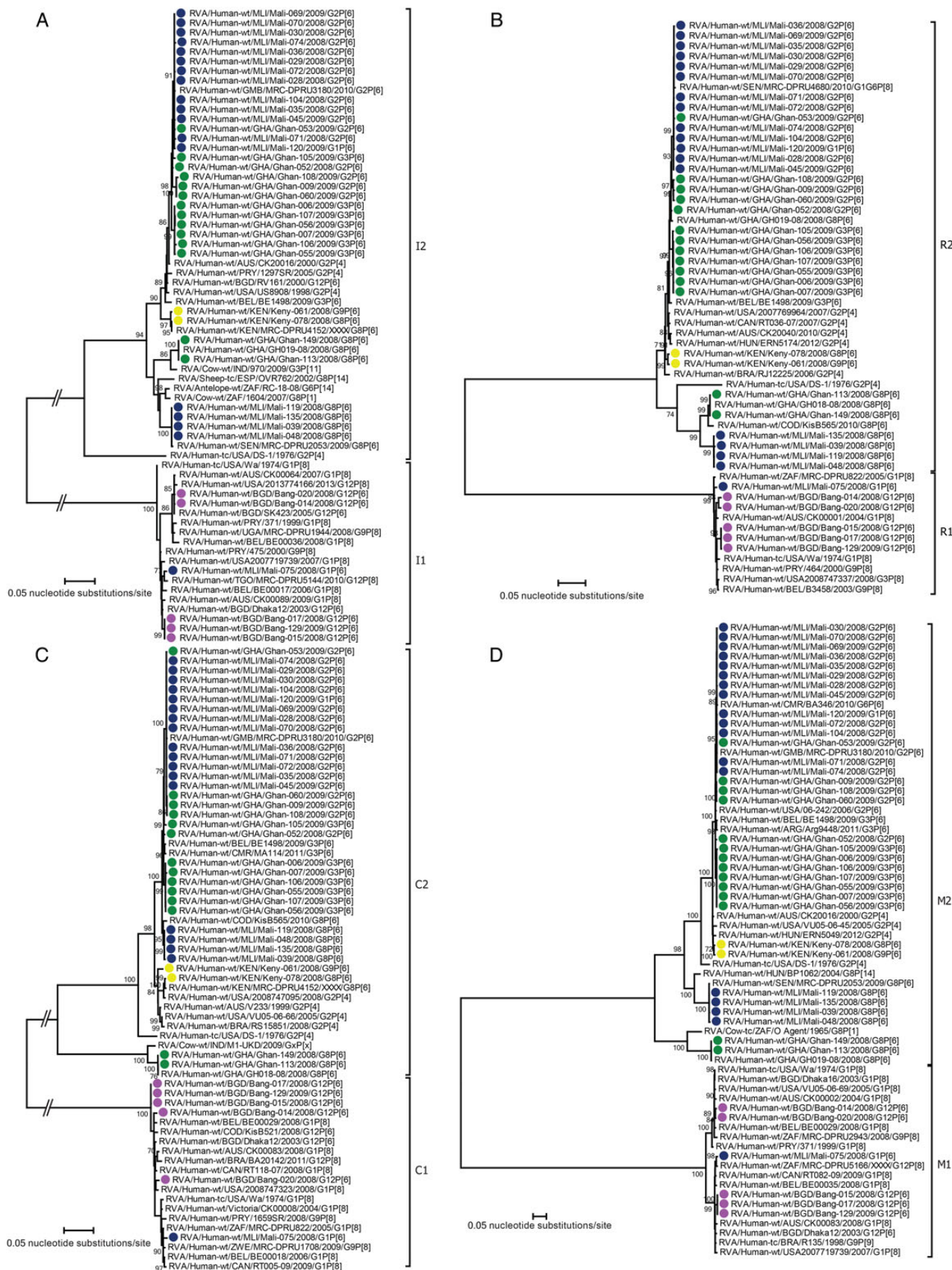


Figure 4. Phylogenetic dendrogram based on the nucleotide sequence of the VP6 (A) and VP1-3 (B-D) genes. Bootstrap values (500 replicates) of >70 are shown. Rotaviruses analyzed in this study are indicated with a circle color coded according to country of origin. This figure is available in black and white in print and in color online.

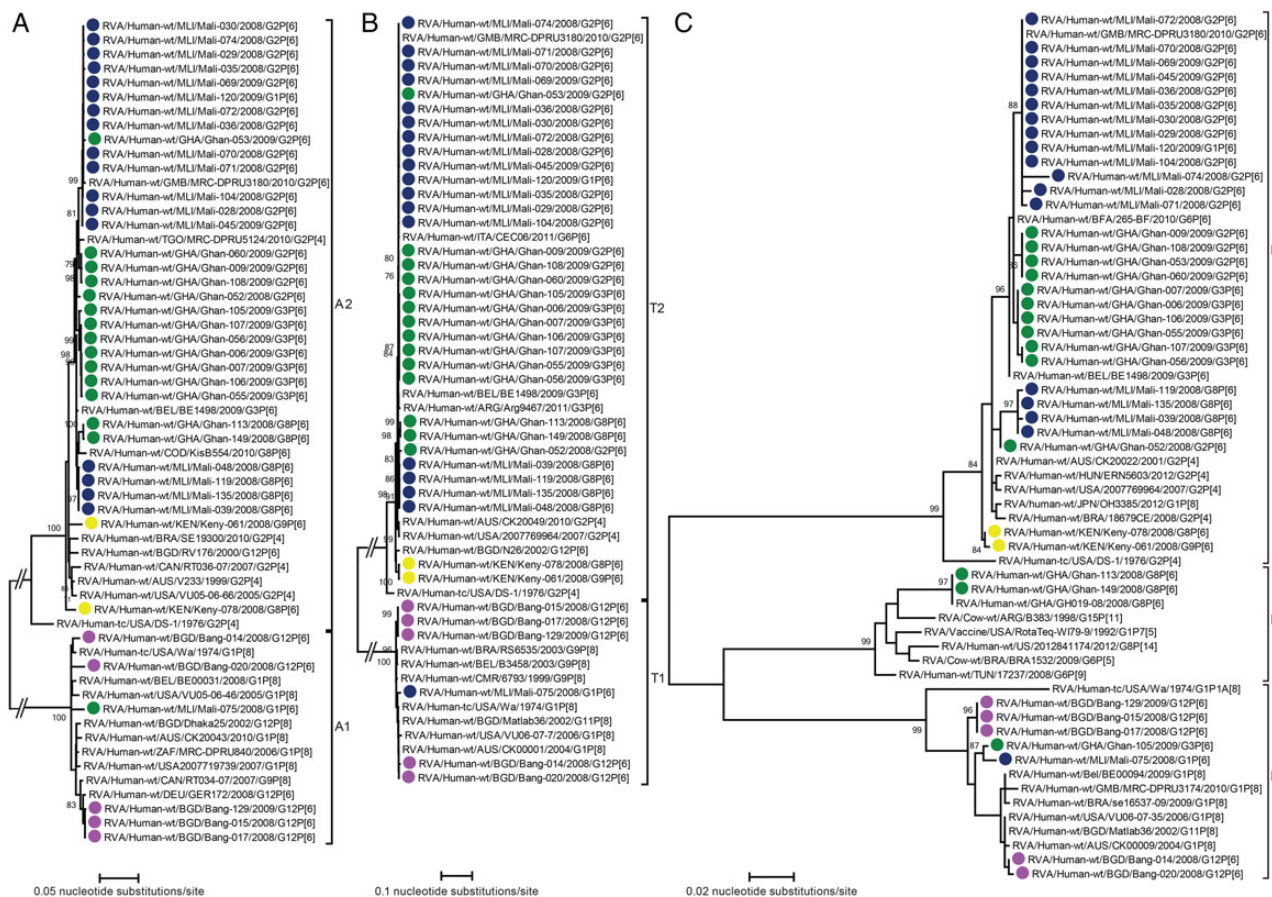


Figure 5. Phylogenetic dendrogram based on the nucleotide sequence of the NSP1 (A), NSP3 (B), and NSP5 (C) genes. Bootstrap values (500 replicates) of >70 are shown. Rotaviruses analyzed in this study are indicated with a triangle color coded according to country of origin. This figure is available in black and white in print and in color online.

within the N1 genotype, together with contemporary human Wa-like rotaviruses isolated around the world.

Interestingly, the NSP2 N2 genotype contained 4 clusters, one containing the majority of Ghanaian strains and the Kenyan strains clustering closely to human rotavirus strains, possessing a typical DS-1-like genotype constellation. The remaining 3 clusters included (1) the majority of closely related Malian NSP2 gene segments, together with Ghan-053; (2) all 4 Malian G8P[6] strains; and (3) the 2 Ghanaian G8P[6] strains. All analyzed strains of these 3 clusters were only distantly related to typical human rotavirus strains, suggesting an animal origin of these NSP2 gene segments.

The remaining gene segments, VP6, VP1–3, NSP1, NSP3, and NSP5, showed a similar clustering pattern for all 32 non-G8 P[6] strains. As illustrated in Figures 4 and 5, these 32 rotaviruses were found either in combination with genotype 1 or with genotype 2. Only within the NSP5 tree (Figure 5C) was an additional genotype found (H3) for the 2 G8P[6] strains from Ghana (Ghan-113 and Ghan-149). For each of the genotype 2 clusters in the phylogenetic trees of the gene segments VP6, VP1–3, NSP1, NSP3, and NSP5, a west/east cluster

deviation was shown for the African strains, although only minor variations were noted between the strains isolated in this study. For all these genotypes (I2, R2, C2, M2, T2, and H2), a close phylogenetic relationship was found between our strains and contemporary human DS-1-like strains, without evidence for animal rotavirus gene segments.

For all 7 gene segments, the genotype 1 cluster contained all 5 strains isolated in Bangladesh, together with strain Mali-045, the only African strain found in this study with a complete Wa-like background. Within the H1 genotype, 2 additional strains were found, Ghan-105 and Keny-061, both possessing an H1 gene segment in a DS-1-like background. Overall, the analyzed strains clustered closely to other contemporary human Wa-like strains isolated around the globe.

DISCUSSION

This study describes the largest data set of complete P[6] rotavirus strains, isolated in a low-income country setting, as well as in a clinical trial setting, thus far. In total, 39 P[6] strains were completely sequenced using next-generation sequencing techniques, showing a large diversity within the G genotypes. This

observation indicates that reassortment events involving the VP7 gene segment frequently happen. A clear example of this involves the 2 strains Mali-075 and Mali-120, both of which contained highly similar G1P[6] genotypes in combination with a completely different genotype constellation (Figures 1 and 2). In contrast to the large genotype diversity found within the VP7 gene, only limited diversity was observed within the genetic backbone of the analyzed P[6] strains. The majority of strains possessed a genetic backbone belonging to one of two typical rotavirus genotype constellations found in humans worldwide, Wa-like (I1-R1-C1-M1-A1-N1-T1-E1-H1) or DS-1 like (I2-R2-C2-M2-A2-N2-T2-E2-H2). Only a limited amount of reassortment events between DS-1-like and Wa-like gene segments were observed, more specifically in the nonstructural NSP2 and NSP5 genes. Interactions between NSP2 and NSP5 are required to form viroplasms, cytoplasmic inclusions in which rotavirus genome replication occurs [54]. Evidence for intergenogroup reassortment between Wa-like and DS-1-like human rotaviruses for both NSP2 and NSP5 genes have been reported in previous studies, indicating that NSP2 and NSP5 genes are more prone to be exchanged between different rotavirus strains [20, 55]. One study even described the presence of a vaccine-derived NSP2 gene that had reassorted into human Wa-like rotaviruses [56]. These observations highlight the fact that reassortment events between vaccine strains and circulating human strains are likely to happen in the future, stressing the importance of continued surveillance both before and after vaccine introduction. However, no vaccine-derived gene segments were found in this study.

The data from this study suggest the circulation of completely unrelated P[6] rotavirus populations in Asia and Africa. All P[6] rotavirus strains isolated in Bangladesh possessed a complete Wa-like backbone and were found together with G12, while 26 of 27 rotavirus strains from sub-Saharan Africa were found together with a DS-1-like genotype constellation and G1, G2, G3, or G9. The limited number of complete P[6] genomes available from Asia and sub-Saharan Africa thus far does not allow us to further investigate whether this observation is an artifact of the selected samples or a more general fact. Additional large sequencing studies in low-income countries are needed to draw more-general conclusions. With the recent advancements in next-generation techniques, making it possible to sequence larger amounts of genomes at a reasonable cost, this aim becomes more and more feasible.

Interestingly, several of these P[6] rotavirus strains (in combination with G1, G2, or G9) appeared to have a single gene segment (NSP2 or NSP4) with a potential bovine-like rotavirus origin (Table 1), in contrast to the high number of gene segments of animal origin found within G8P[6] rotaviruses [52]. However, potential bovine-like rotavirus gene segments have not exclusively been encountered in rotavirus strains carrying the P[6] genotype, but have also been found in rotavirus strains

carrying the P[4] and P[8] genotypes cocirculating during the clinical trial (data not shown). In addition, potential bovine-like rotavirus gene segments have been found in non-P[6] strains isolated in a number of countries worldwide, including Brazil, Pakistan, Iraq, the Democratic Republic of the Congo, Australia, and Belgium [57–60].

Taken together, these data suggest that the genetic backbone of the vast majority of the P[6] rotavirus strains circulating in Mali, Ghana, Kenya, and Bangladesh are very similar to the worldwide cocirculating P[4]/DS-1-like or P[8]/Wa-like rotavirus strains [20]. Therefore, it is unlikely that the difference in vaccine efficacy between high- and low-income countries is the result of a different genetic composition of circulating rotavirus strains. However, based on the complete genomes determined in this study, the VP4 gene segment belonging to the P[6] genotype seems to be the main determinant of whether certain strains are able to rapidly spread worldwide among the human population over longer periods. Therefore, it is likely that the P[6] segment by itself may be responsible for the restriction of these strains to the African and Asian continents. One plausible explanation for this observation would be the difference in histo-blood group antigens phenotypes present in white and nonwhite populations. Further investigation is needed to determine whether histo-blood group antigens can be related to host susceptibility/resistance factors for (P[6]) rotaviruses, as been proven to be the case for susceptibility to norovirus infection [61]. Potentially, the number of susceptible people (eg, people who synthesize the H-type 1 antigen) outside Africa and Asia might not be high enough for P[6] strains to become epidemiologically important and successfully compete with P[4] or P[8] strains.

Notes

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